

## Differential susceptibility of fresh *Trichomonas vaginalis* isolates to complement in menstrual blood and cervical mucus

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**SUMMARY** The ability of complement in human menstrual blood and cervical mucus to kill *Trichomonas vaginalis* was compared with that of complement in serum, and 95 fresh trichomonal isolates obtained from vaginal wash material were evaluated for susceptibility to complement immediately after isolation. Only serum and menstrual blood with haemolytic activity produced total cytolysis of *T vaginalis*. The cytolytic abilities of these fluids were totally inactivated by treatment with heat or edetic acid (EDTA), which confirms the role of complement in cytolysis. Most cervical mucus samples had no detectable trichomonal cytotoxic properties. The cytotoxic activity in the remaining samples was not due to complement, as it was heat stable. Fresh isolates of *T vaginalis* and subpopulations of fresh isolates differed in their susceptibility or resistance to complement mediated lysis in serum. Resistance to complement did not remain stable after trichomonads were grown in vitro.

*Trichomonas vaginalis* is a sexually transmitted urogenital parasite. The natural environment of *T vaginalis* is the cervicovaginal mucus, which is enriched periodically by menstrual blood. The mucosal parasite is not in contact with the bloodstream under normal conditions.

The considerable lytic effect of fresh non-immune vertebrate serum on *T vaginalis* has been known for many years.<sup>1-3</sup> The factor responsible for lysis has been identified as activation of the alternative pathway of complement by *T vaginalis* in the absence of specific antibodies.<sup>4,5</sup>

Investigations have shown great variability in the surface protein and carbohydrate molecules of the parasite.<sup>6-16</sup> Plasticity of the surface membrane may be an important factor influencing the susceptibility or resistance of *T vaginalis* to host immune factors.

We performed this study to characterise further the role of complement in the lysis of fresh *T vaginalis* isolates, to investigate whether menstrual blood and cervical mucus have complement that can destroy the parasite in vitro, and to ascertain whether different

isolates had variable susceptibility to complement cytolysis.

### Patients, material, and methods

#### SAMPLES FROM PATIENTS

We studied 119 patients (aged 16 to 53) with trichomoniasis, either symptomatic or symptomless. Purulent discharge, erythema of the vaginal wall, and vulvovaginal irritation were the most common clinical signs in symptomatic patients. In women with regular menstrual cycles the phase of the cycle was recorded, when possible.

Normal human serum was obtained from adults with no history of trichomoniasis, pooled, stored at -20°C in 1 ml aliquots, and thawed immediately before testing. The haemolytic activity of the pooled serum was found to be within the normal range.

Samples of menstrual blood from the second and third days of bleeding were obtained from gynaecological patients without trichomoniasis and from healthy volunteers. Menstrual blood was collected in plastic containers (40 × 60 mm) applied to the introitus of the vagina by individual women on waking in the morning. Immediately after delivery the samples were centrifuged at 800 × g for 10 minutes, and the supernate from menstrual blood was either tested

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immediately or stored at  $-20^{\circ}\text{C}$ . Menstrual blood was obtained from six patients by vaginal cups placed on the tip of the cervix for two hours.

Specimens of cervical mucus were obtained at different phases of the menstrual cycle from 112 women attending a gynaecological outpatient department at the Institute of Parasitology. The samples were collected through a sterile plastic tube attached to a syringe. Material was either assayed immediately or stored in 1.5 ml polyethylene microfuge tubes at  $-20^{\circ}\text{C}$  for later use.

#### ORGANISMS

We used a *T vaginalis* strain called DD-3 as the standard laboratory strain throughout the optimisation of the cytolytic experiments and to provide a positive control in each assay. It had been isolated from material from the posterior fornix of the vagina of a woman age 31 who had symptomatic trichomoniasis. Axenisation was achieved by three transfers in Diamond's TYM (trypticase, yeast extract, and maltose) medium<sup>17</sup> supplemented with 10% bovine serum, penicillin 1000 IU/ml, and streptomycin 1000  $\mu\text{g}/\text{ml}$ . Cultures had been grown in vitro for about two years. Two to three transfers in agar free TYM medium were performed before use in all experiments. Only cultures in the late logarithmic phase were used in the tests. The cells were washed three times by centrifugation at  $800 \times g$  for 10 minutes in phosphate buffered saline (PBS) pH 7.2, and were resuspended in PBS to a density of  $4 \times 10^5$  cells/ml.

We obtained fresh trichomonal isolates from vaginal washes from 95 patients with trichomoniasis. Sterile isotonic saline (10 ml) was instilled into the posterior fornix of the vagina by a syringe attached to a polyethylene tube. The vaginal wash material was examined immediately. Trichomonads were washed three times at  $250 \times g$  for 10 minutes in PBS before being resuspended to  $4 \times 10^5$  organisms/ml to test the susceptibility of the parasites to complement lysis. Strain DD-3 tested with 10% serum was always included as a positive control. Isolates were axenised by subsequent transfers in the TYM complex medium supplemented with 10% bovine serum and antibiotics. Cultures were also continuously passaged in antibiotic free TYM and serum medium to monitor the complement lysis later. All isolates were cryopreserved in liquid nitrogen.

#### CYTOTOXICITY ASSAYS

Various dilutions of menstrual blood or serum were mixed with an equal volume of  $4 \times 10^5$  *T vaginalis*/ml and incubated for 30 minutes at  $37^{\circ}\text{C}$ . Serum or menstrual blood that had been heat inactivated at  $56^{\circ}\text{C}$  for 30 minutes or treated with 10 mmol/l EDTA were tested at the same time. Controls of *T vaginalis*

with PBS were included. The remaining motile parasites were counted under microscopy in a haemocytometer, and the extent of cytotoxicity was expressed as percent lysis using the following formula:

$$\frac{\text{No of motile parasites in control} - \text{No in sample}}{\text{No of motile parasites in control}} \times 100$$

All experiments were performed in triplicate.

The ability of cervical mucus to kill trichomonads was assayed by mixing thoroughly  $5\mu\text{l}$  containing  $2 \times 10^4$  *T vaginalis* with  $15\mu\text{l}$  cervical mucus applied by  $5\mu\text{l}$  Partigen dispenser (Behring). Preparations were covered by a cover slip and incubated at  $37^{\circ}\text{C}$  in a moist chamber for one hour. Viability of the parasite was assessed semiquantitatively by microscopic evaluation and expressed as 100% viability of organisms, 25% to 75% killing of the parasites, or 100% killing of trichomonads. Mucus samples expressing 100% cytotoxic activity were examined again after EDTA treatment and after heat inactivation.

#### MEASUREMENT OF HAEMOLYTIC COMPLEMENT ACTIVITY

Complement activity (expressed in  $\text{CH}_{50}$  units) was measured by a modification of the standard haemolytic assay. Because of the lower concentration of haemolytic complement in menstrual blood than in serum, a starting dilution of 1:25 was used for menstrual blood instead of the 1:50 used for serum.

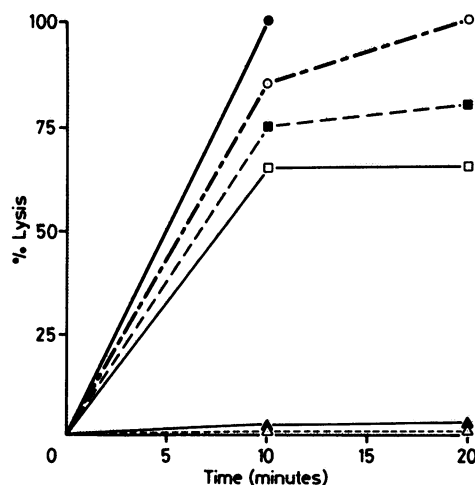


Figure Representative complement mediated lysis of *Trichomonas vaginalis* strain DD-3. About  $2 \times 10^5$  washed motile organisms were incubated with 50% (●), 10% (○) 5% (■) and 2.5% (□) pooled normal human serum at  $37^{\circ}\text{C}$ . Parasites were also incubated in 50% pooled normal human serum heat inactivated (▲) or treated with 10 mmol/l edetic acid, (△) lysis was calculated using the formula:

$$\frac{\text{No of motile parasites in control} - \text{No in sample}}{\text{No of motile parasites in control}} \times 100$$

Table 1 Measurement of complement mediated haemolysis (CH) in menstrual blood of 50 women without trichomoniasis

Complement activity (CH <sub>50</sub> /ml)*	No (%) of samples showing given activity (n = 50)
0	15 (30)
0.1-1.0	3 (6)
1.1-10.0	17 (34)
10.1-22.0	15 (30)

\*Complement activity assessed by haemolysis of sheep red blood cells.<sup>18</sup> Venous blood serum samples tested at the same time gave haemolysis of 30-40 CH<sub>50</sub>/ml.

## Results

### MEASUREMENT OF COMPLEMENT ACTIVITY

Strain DD-3 was chosen initially for optimisation of the experimental conditions because of its high susceptibility to lysis by serum complement. The figure shows the pattern of cytotoxicity of *T vaginalis* DD-3 with pooled normal human serum. The pooled normal human serum possessed a complement activity of 30-40 CH<sub>50</sub>/ml, which was consistent with reported values. Treatment with heat or EDTA totally inactivated the lytic activity of the serum, which indicated that complement played a part in killing the parasite.

We then wanted to know the relative amount of haemolytic activity of complement in menstrual fluid to assess whether this fluid could also produce complement mediated parasite lysis. As can be seen in table 1, maximum haemolytic activity in menstrual blood was only about half that of venous blood, and 30% of menstrual blood samples had no haemolytic complement. As expected for this assay, treatment with heat or EDTA destroyed the ability to cause haemolysis.

### EFFECT OF MENSTRUAL BLOOD COMPLEMENT ON *T VAGINALIS*

We performed experiments to measure complement mediated cytotoxicity of menstrual blood for *T vaginalis*. Table 2 shows that the total destruction of all parasites (100% lysis) occurred only in menstrual blood with active haemolytic complement. We found

Table 2 Relation between complement mediated sheep red blood cell haemolysis and lysis of *Trichomonas vaginalis* strain DD-3 by menstrual blood of 50 women without trichomoniasis

Haemolytic complement in menstrual blood (CH <sub>50</sub> /ml)*	No of samples	No of samples causing lysis of <i>T vaginalis</i>	% lysis of <i>T vaginalis</i>	
			Mean	Range
0	15	3	9	0-34
0.1-10.0	20	20†	63	1-100
10.1-22.0	15	15†	99	98-100

\*As described in Table 1.

†Heat inactivation at 56°C for 30 minutes or treatment with 10 mmol/l edetic acid (EDTA) totally destroyed lytic effect of menstrual blood. Lysis calculated using formula:

$$\frac{\text{No of motile parasites in control} - \text{No in sample}}{\text{No of motile parasites in control}} \times 100$$

Table 3 Differentiation between 95 *T vaginalis* isolates by their susceptibility to complement mediated lysis

Concentration of serum (%)	No of isolates lysed (percent lysis)		
	0-50%	51-99%	100%
10	8	44	43
50	1	15	79*

\*Including the 43 isolates killed totally at 10% serum concentration.

that the lytic effect of the samples collected direct from the cervix was similar to that of those obtained from the vagina. The weak trichomonacidal effect of the three samples with no measurable haemolytic complement was not inactivated by heat or treatment with EDTA.

### EFFECT OF CERVICAL MUCUS ON *T VAGINALIS*

Cytotoxicity of cervical mucus was very low compared with that of the serum from venous and menstrual blood. Of the 112 mucus samples, 95 had no effect on *T vaginalis* viability, 11 showed a moderate degree of cytotoxicity (25%-75% of the parasite population killed), and only six completely destroyed all trichomonads. In those six samples, however, heat inactivation or treatment with EDTA did not abolish the killing activity, which indicated a role of other non-complement factors in mucus in the destruction of parasites.

### DIFFERENTIATION BETWEEN *T VAGINALIS* ISOLATES ON THE BASIS OF COMPLEMENT MEDIATED CYTOLYSIS

The effect of serum complement on 95 fresh isolates was then investigated. As shown in table 3, individual isolates differed noticeably in their sensitivity to complement lysis. Only 43 of the 95 isolates were totally destroyed in a 10% concentration of serum. The percentage survival of the remaining isolates in 10% and 50% serum varied widely. No relation was found between the susceptibility of a given isolate to complement lysis and the phase of the menstrual cycle at the time of its isolation; similarly, no relation was found between the susceptibility of an isolate and the severity of infection of the woman from whom it had been isolated. Heat inactivation and treatment with EDTA abolished the cytolytic effect of serum on *T vaginalis*. The sixteen isolates that survived in 50% serum were tested later for their resistance to complement. None retained its resistance after being grown in vitro for one or two weeks.

## Discussion

*Trichomonas vaginalis* is a protozoan parasite known to be susceptible to the cytolytic effect of serum complement.<sup>1-3</sup> In its natural environment, however, the micro-organism is not in contact with venous blood complement, except possibly during menstruation.

Little is known about the complement activity of cervical mucus, though some of the components of complement have been found in this fluid.<sup>19</sup> We have been unsuccessful in finding any published data concerning direct activity of menstrual blood or cervical mucus on *T vaginalis*. We therefore wanted to test whether complement was in fact present in the fluids of the urogenital region and to examine the susceptibility of fresh trichomonal isolates to this complement.

The results of our present study (tables 1 and 2) show that complement activity in menstrual blood, though surprisingly low compared with that in serum, may indeed contribute to parasite destruction in vitro. During investigations of patients with trichomoniasis we have recorded a reduction of the parasite numbers in the vagina of some patients during menstruation.<sup>20</sup> Our results also suggest that complement present in menstrual blood may be responsible for in vivo parasite killing.

Our finding of the overall lack of trichomonacidal effect of cervical mucus samples confirms Schumacher's conclusions about the almost total absence of complement activity in this material.<sup>19</sup> The destructive effect of some mucus samples, which persists after heat inactivation, suggests that other non-complement factors participate in parasite killing by mucus in vitro.<sup>21</sup>

Organisms were tested for sensitivity to complement mediated cytolysis immediately after their recovery from the vagina. Our data suggest variable resistance to lysis in normal serum by fresh isolates (table 3). Differences in sensitivity to complement occurred during in vitro growth and in isolates successively taken from patients at different phases of their menstrual cycle (data not shown). Even in a given isolate, the variable sensitivity of individual parasites to complement cytolysis was obvious, as shown by different survival rates of trichomonad populations in 10% and 50% serum (table 3). These results strongly indicate possible dynamic changes of the parasite surface membrane. It will be important to know whether isolates and their subpopulations, as differentiated on the basis of susceptibility to complement lysis, correlate with the defined surface markers used to study the phenotype of the pathogenic human trichomonads.<sup>9,21</sup>

Mechanisms for avoiding destruction by complement or some other immune factors seem to be an adaptive necessity for *T vaginalis*, which is in periodic contact with immune factors during menstruation. The results of this work indicate that resistance to complement lysis may present a sensitive indicator of certain membrane changes of *T vaginalis* that permit successful parasitism of the host mucosa.

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